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Screening for antifungal activity of endophytes isolated from Burseraceae

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Contenido

INTRODUCCION

La presente tiene por objeto brindar una explicación sobre el contenido y diseño de los documentos que comprenden esta tesis como requisito de grado del Programa de Maestría en Biología Aplicada de la Universidad Militar Nueva Granada.

Desde el grupo de investigación InQuiBio en sus trabajos recientes, se ha forjado un nuevo camino en el hecho de reformar el formato tradicional de presentación de trabajos de grado, de un documento tipo libro a un formato por capítulos basados en los objetivos del trabajo, lo que permite un mayor acercamiento a la forma de presentación de artículos científicos en ingles, facilitando así la transición de los trabajos derivados de las tesis de grado a productos científicos específicos.

Con estos antecedentes, los directores y el estudiante decidieron conjuntamente presentar los resultados de la tesis como artículo científico, divido en dos documentos derivados del desarrollo de la investigación. Con esto también se facilita la lectura y la evaluación del trabajo desarrollado por el estudiante.

<u>CAPITULO 1</u>

Screening of fungal endophytes isolated from *Protium heptaphyllum* and *Trattinnickia rhoifolia,* as antagonists of *Fusarium oxysporum.*

Abstract

Control of fungal pathogens is mainly addressed by chemically synthesized fungicides which, after a prolonged use, produce environment pollution and develop resistance. In this context, endophytes have been recognized as potential biocontrolers and also potential source of antifungal metabolites. Therefore, as part of our research on phytopathogen controllers, 355 fungal endophytes were isolated from *Protium heptaphyllum* and *Trattinnickia rhoifolia* (Burseraceae), both important etnobotanical tree species that produce secondary metabolites of agronomic and industrial interest. Endophytes were tested by *in vitro* dual culture against *Fusarium oxysporum,* an important phytopathogen of agronomic importance. Five endophytes exerted at least 40 % of inhibition on *F. oxysporum* growth. Ethyl acetate (EtOAc) extracts were obtained from the most-antagonistic fungi growth in three different liquid media. The extracts were tested against a conidial suspension of *F. oxysporum* by direct bioautography. Two extracts derived from fungi identified as *Chaetomium globosum,* F211_UMNG and *Meyerozima* sp. F281_UMNG exhibited inhibition of the pathogen growth. The isolate *Chaetomium globosum,* F211_UMNG was selected for a chemical profiling by RP-HPLC-DAD-ESI-MS and antifungal molecules such as cladosporin, chaetoatrosin A and chaetoviridin A were tentatively identified by their MS data.

Keywords: *Endophytes, Burseraceae, Chaetomium, metabolites.*

Introduction

Using chemically synthesized fungicides has been the first line strategy to control, phytopathogenic fungi (Oerke, 2006). However, secondary effects due to the use of these products, such as environment pollution and resistance development, had leaded to a growing reluctance for the use of hazardous fungicides in agriculture. Thus, an enhanced trend in searching new control strategies involving environment friendly alternatives in the management of plant pathogens has arisen (Kidane & Laing, 2008). In the search for such control strategies, naturally occurring chemical entities result as potential alternatives for the industry to replace synthetic products (Kusari et al., 2014). In this context, microorganisms have resulted a rich source of compounds with useful properties (Zaher et al., 2015) for several applications in the agrochemical and pharmaceutical industries (Ezra et al., 2004; Kusari et al., 2014).

Over several decades, interaction between fungal endophytes and their hosts has been attracting attention of researchers because of the advantageous characteristics they confer to plants, such as enhanced stress tolerance, plant growth factors production, herbivores repellency and protection against pathogens, among others (Kanchiswamy et al., 2015). This last is due, at least in part, to the fact that endophytes compete with other microorganisms for a specific niche and this could be achieved by the production of antibiotics-like secondary metabolites, along with other strategies (Arnold et al., 2003). As a consequence of their repellent properties, endophytes have been proposed as biocontrolers or source of antifungal metabolites against phytopathogens of agronomic importance (Kanchiswamy et al., 2015).

As part of our ongoing search for biologically active secondary metabolites from endophytic fungus, the objective of this work was to explore the diversity of endophytes isolated from *Protium heptaphyllum* and *Trattinnickia rhoifolia* (Burseraceae) form Casanare, Colombia. These tree species have been traditionally used by indigenous communities to treat several ailments (Gompper et al., 1993), and their complex chemical repertory has provided useful compounds with industrial, pharmaceutical and agronomical potential (Siani et al., 2012; Violante et al., 2012). Also from a species in the Burseraceae family, endophytes has been isolated with demonstrated potential for controlling phytopathogens, as *Muscodor yucatensis* (Macías-Rubalcava et al., 2010). Therefore, the aim of this work was to test *in vitro* the abilities of the endophytes to inhibit, by metabolite production, the mycelial growth of *Fusarium oxysporum.* Which is a pathogen of many plant species, and represent a major treat for production of several agriculturally important crops, such as banana, carnation, tomato, dates, chickpeas, lentils, and others (Michielse & Rep, 2009). The active component, or components, responsible of the antifungal activity were partially characterized following a bioassay-guided fractioning test, for the liquid culture-derived crude extract of the most antagonistic endophyte, to be incorporated in the future to a control management programs for the plant pathogen *F. oxysporum.*

Methods

Recover of endophytes and isolation

A total of two individuals from *Protium heptaphyllum* and two form *Trattinnickia rhoifolia* were collected at foothill of the west Colombian Andean mountains in Aguazul, Casanare, Colombia (N 05°13'47.89", W 072°30'31.38"), a transition ecosystem between the savanna and the high Andean ecosystems. Botanical specimens of *P. heptaphyllum* (Aubl.) Marchand (COL573961) and *T. rhoifolia* (Aubl.) Marchand (COL573962) were deposited in the Colombian National Herbarium. From ach tree, plant material, from higher, medium and lower strata was sampled. Five leaves per level were collected in a total of 60 leaflets that were bagged in sealed bags and stored in dark and conditions for 24 h. Plant material was vigorously washed with sterile water and Tween 20 (0.01%), then submerged in 70% aqueous ethanol (1 min), then in 1% sodium hypochlorite (3 min), and then rinsed three times with sterilized water. Leaves were then imprinted on Potato Dextrose Agar (PDA, Oxoid, UK) to verify for disinfection of all epiphytic microorganisms.

Each leaf was sectioned into 2 mm^2 pieces, and 5 randomly-choose pieces from each leaf were seeded in Petri dishes (90 x15 mm^2) containing Water Agar (agar 1.5%) (WA), 1/10 PDA (PDA at a 10^{th} of the recommended concentration) or PDA, and then incubated at 26 ºC. Hyphae tips emerging from the leaf pieces were collected for three weeks, and subcultured on PDA. Axenic cultures were established and when a particular fungus sporulated, a monosporic culture was established. A hypha tip culture was obtained if no sporulation occurred.

Identification of endophytes

Fungal populations were identified on the basis of cultural characteristics and morphology of fruiting bodies and spores (Barnett, & Hunter, 1998; Hanlin, 1998; Kiffer & Morelet, 2000). Fungi were identified up to the genus level by observing the presence of conidial mycelium, spore mass color, distinctive reverse colony color, production of diffusible pigments, and spore morphology (Barnett, & Hunter, 1998). Cultures that repetitively failed to sporulate on different media were recorded as *mycelia sterilia*.

Additionally, those endophytes that inhibited of *F. oxysporum* growth above 40 % were identified by amplification the Nuclear ribosomal internal transcribed spacer (ITS) region, using the primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS 4(5'-TCC TCC GCT TAT TGA TAT GC-3') (White, 1990). Amplicons were sequenced with the same primers bidirectionally a single time in Macrogen inc. (Korea) and the result sequenence were aligned and edited in BioEdit v7.2.5 (Hall, 1999). The sequences were confronted with the GeneBank database (http://www.ncbi.nlm.nih.gov), using BLASTN 2.2.28 (Zhang et al., 2000). The closest match was selected and aligned using Clustal W (Thompson et al., 1994). For phylogenetic analysis, tree constructions were done with the MEGA 6.0 program package (Tamura et al., 2013) with the neighbor-joining method (Saitou and Nei, 1987). Bootstrap analysis was done using 1000-times resampled data. The resulting sequences were deposited in the GenBank.

Ethyl acetate (EtOAc) extraction

Selected fungi were reactivated in 500 mL of Potato Dextrose Broth (PDB, Oxoid, UK), Sabouraud Broth (SAB, Oxoid, UK) and Yeast extract sucrose media (YES) (Keller & Turner, 2012), and cultured in an orbital shaker under constant agitation (100 rpm) at 21ºC for 7 days. After that period the culture was filtered using Whatman No. 1 qualitative filter paper, and the mycelia lyophilized. Separately, both mycelia and filtered media, were mixed with EtOAc in a 1:3 proportion and incubated in an orbital shaker in constant agitation (100 rpm) for 48 h. Organic phase (EtOAc) was separated from the mycelia by vacuum filtration using Whatman No. 1 qualitative filter paper, and from filtered liquid media using a decantation funnel. The resulting extracts were concentrated by liophylization.

Antifungal assays

Fungal endophytes, and a phytopathogenic isolate (form the collection of the phytopathology laboratory from the Universidad Militar Nueva Granada): *F. oxysporum* G1 isolated form *Physalis peruviana*, were cultured on PDA at 26 ºC during 5 days. In order to detect the possible effect of each endophyte on the phytopathogen growth, dual cultures were settled where each isolate was challenged with *F. oxysporum* G1. Thus, a plug (3mm diameter), obtained from the colonial actively growing edge of the endophyte to be tested, was seeded on PDA, 10 mm away from the edge of a Petri dish (90x10 mm^2). At a spot distance (10 mm) from the diametrally-opposed edge, a similar plug of *F. oxysporum* was seeded. Six days later, the effect of each endophyte on *F. oxysporum* growth was observed and *F. oxysporum* colony radial measurement and distance between colonies was recorded. As a control, a plug of each organism was cultured alone. These experiments were replicated three times.

The antifungal activity of the extracts was tested by direct TLC bioautographic detection (Dewanjee et al., 2015). Extracts and fractions from the selected endophytes (Table 1) were diluted in ethanol (HPLC grade) and 30 mg were seeded in a single spot on a TLC Aluminum silica gel 60 Sheets 20x20 cm (Sigma-Aldrich). Then the silica sheet was sprayed with a $1x10^6$conidia/mL conidial suspension of *F. oxysporum* until the whole sheet was covered. The assays were incubated in a humid chamber for 3 days and were evaluated under UV-light incidence.

Fractionation of the most active extract

The most active extract was fractionated by preparative HPLC using a Shimadzu prominence LC20AD instrument, in gradient elution, using a Shimadzu Premier column C-18 (4,6 x 150 mm, 5 mm) at a flow rate of 2 mL/min. The injection volume was 50 µL. The mobile phases consisted in methanol (HPLC grade) (Phase A) and trifluoroacetic acid 0.005% (HPLC grade) (Phase B). Separation was carried out during 25 min in a

FRC 10A Shimadzu fraction collector. A diode array detector (DAD) performed signal detection at 270 nm. A total of 20 fractions were recovered and were concentrated by lyophilization.

LC-MS-based chemical profiling

Extracts and fractions were characterized by Reverse Phase Liquid Chromatography with multiwavelength UV-VIS detection (by a DAD) and coupled by electrospray to mass spectrometry (RP-HPLC-DAD-ESI-MS). Analyses were performed on a Shimadzu prominence instrument, in gradient elution, using a Shimadzu Premier column C-18 (2.1x150mm, 5mm). Simultaneous monitoring was carried out at 270 nm, at a flow rate of 0.6 mL/min. The operating temperature was 30 °C and the injection volume was 20 µL. As a mobile phase A was used 1% formic acid in distilled water (HPLC grade), and acetonitrile (ACN) (HPLC grade) was used as mobile phase B, and separation was carried out during 33 min. The mass spectrometry detector (MSD) consisted of an electrospray ionization (ESI) and a detector with a triple quadrupole mass analyzer. The mass spectrometry method consisted of a scan in simultaneous positive and negative ionization with an acquisition time of 2-33 min, a mass range of 50-2000 m/z, a scan speed of 1667 u/s, an event time of 0.5 s, nebulizer gas flow of 1.5 L/min, 350 °C interface temperature and DL, and 450 °C block temperature. The drying gas flow rate was 9 L/s. The analysis was monitored at wavelengths between 270 and 330 nm. Tentative identification of the major and minor metabolites in the extract was performed by mass spectra based analysis, complemented with the analysis of reported fungal metabolites.

Results

Recover of endophytes

A total of 577 endophytes were isolated from 900 cultured pieces of leaflets. A sub total of 355 endophytes were selected after elimination of redundant morphotypes derived from the same leaflet. The highest number of endophytes (n=236) was recovered from the lower collection level, for both species (Fig. 1). To determine the phylogenetic relationships of these isolates, the ITS region of these endophytes was amplified and sequenced (Fig. 2). Form a 570 bp sequences, phylogenetic tree was constructed, and isolates clustered as follows: endophyte F92_UMNG clustered with *Talaromyces amestolkiae,* F18_UMNG with *Phyllosticta* sp., F211_UMNG with *Chaetomium globosum,* F299_UMNG with *Xylaria grammica,* and F281_UMNG with *Meyerozyma* sp. (Table.1).

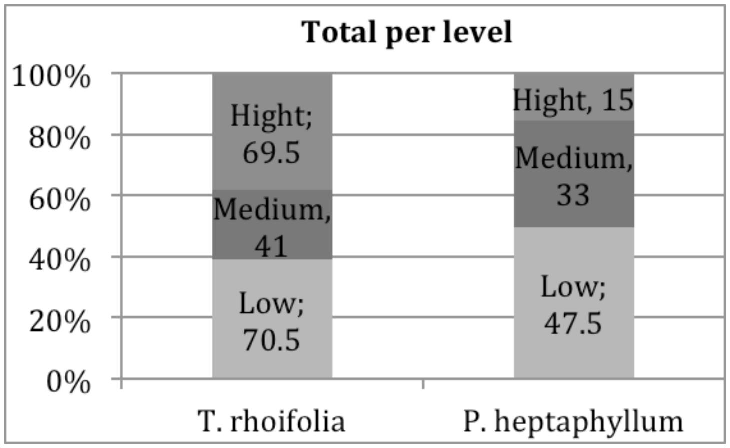

Figure 1. Percentage of culturable fungal endophytes isolates from *T. rhoifolia* and *P. heptaphyllum* at three height levels.

Antifungal test

Antifungal ability of 355 fungal endophytes against *F. oxysporum* G1 was evaluated by dual culture method. Five endophytes that reduced the area of growth of *F. oxysporum* in at least 40%, were found to inhibited the growth of *F. oxysporum,* without colony contact (Fig. 3).

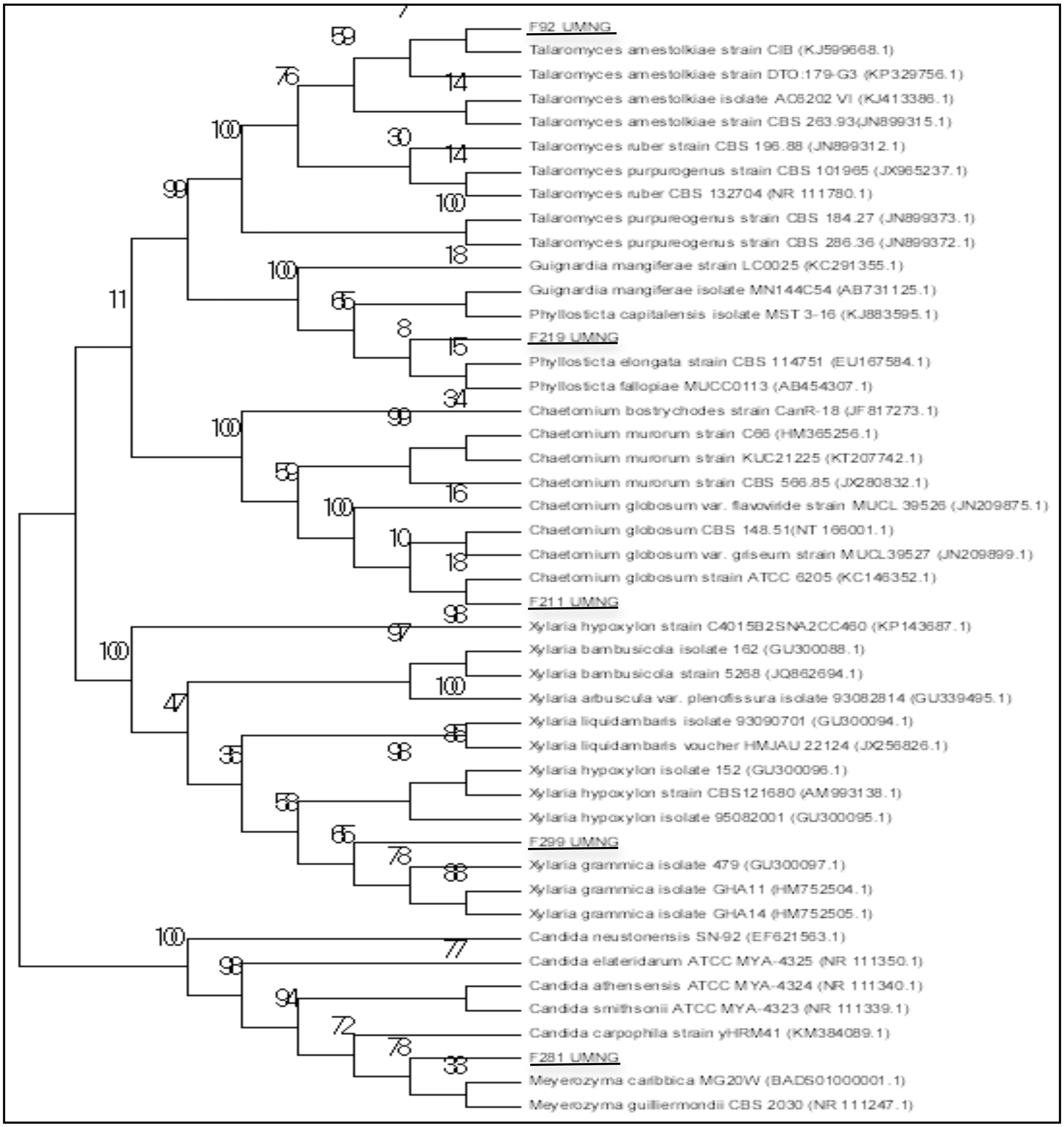

Figure 2. Dendrogram showing the phylogenetic relationship of fungal endophytes based on the ITS region. Phylogenies were inferred using neighborjoining analysis and trees generated in MEGA 6.06 software. Numbers at branch points indicate bootstrap values. The scale bars represent estimated difference in nucleotide sequence. Red underline identified endophytes isolated in this work.

Extracts obtained with EtOAc from two isolates F211_UMNG and F281_UMNG cultured in YES, showed negative effect on *F. oxysporum* radial growth when they were evaluated by bioautography (Fig. 4). When comparing the inhibitory effect on *F. oxysporum* radial growth produced by these two isolates, it was found that isolate F211_UMNG (*C. globosum)* exerted a greater *in vitro* inhibition of the colony growth, arresting it at 12.5 +/- 0.6 mm from the endophyte colony extreme. Meanwhile, F281_UMNG arrested the colony growth at 5.7+/-0.9 mm from the endophyte and represent an inhibition of 64% and 45% respectively (Fig. 3 and 4). Consequently, the extract from F211_UMNG isolate was fractionated in order to determine the fraction in which the inhibitory compound may be found. A total of 20 fractions were recovered (Fig. 6) and were tested by bioautography against a conidial suspension of *F. oxysporum*. Only fraction #14 exerted inhibition of the fungus (Fig. 5) in a quantity of 30 mg.

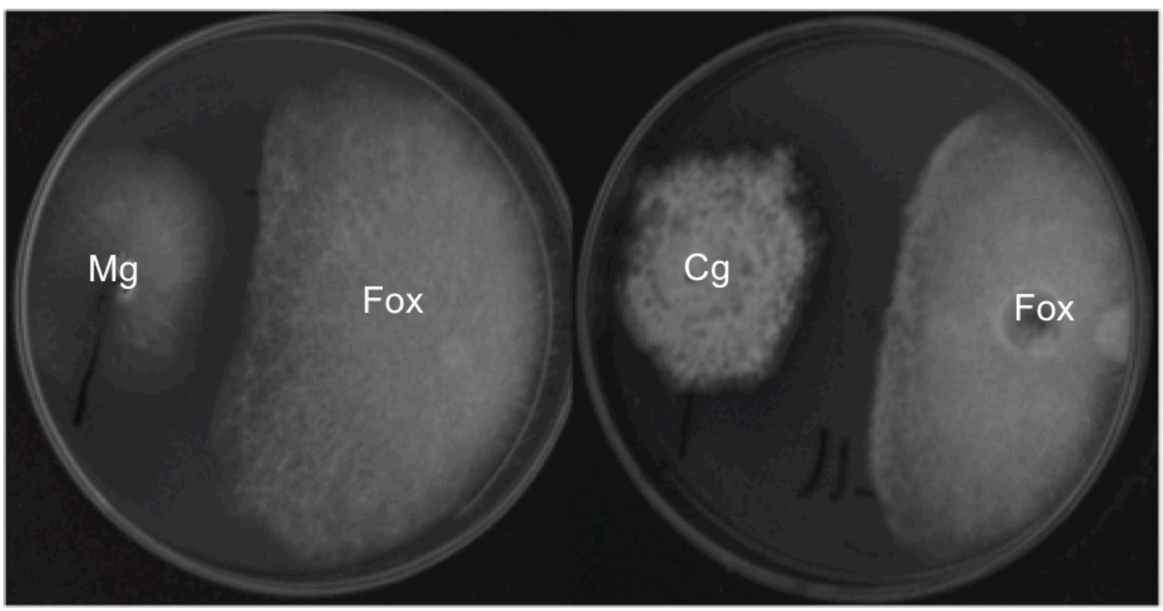

Figure 3. Inhibition of *F. oxysporum* G1 (Fox) produced by *C. globosum* F211_UMNG (Cg) and *Meyerozima* sp. (Mg) in PDA media in dual cultures after 6 days post inoculation.

Fraction #14 was analyzed by LC-MS, rendering a chromatogram that included three defined signals between the 12 and 17 minute (peak 1-3 Fig. 7)(1-3).

Table 1. Isolation media and closest match in phylogenetic analysis by neighbor joining method form the most active endophytes against *F. oxysporum*. Species were defined when the node was supported with <- 90 (Fig. 2).

Endophyte isolation conditions					
Code	**Plant**	**Level**	**Recovered from**	**Closest match**	**NCBI accession**
92	*T. rhoifolia*	Low	PDA 1/10	*Talaromyces amestolkiae*	KU184613
211	*P. heptaphyllum*	Low	WA	*Chaetomium globosum*	KU184610
219	*P. heptaphyllum*	Low	PDA 1/10	*Phyllosticta* sp.	KU184614
281	*P. heptaphyllum*	High	WA	*Meyerozyma* sp.	KU184611
299	*P. heptaphyllum*	Low	WA	*Xylaria grammica*	KU184612

Discussion

Alternative bioagents and molecules to synthetic fungicides are needed to control the vascular wilt pathogen *F. oxysporum*, which is a major limiting factor of several agronomicallly-important crops. In the present study we isolated 577 fungal endophytes from *P. heptaphyllum* and *T. rhoifolia* were a subset of 355 endophytes was selected after elimination of redundant morphotypes. The endophytes were recovered in three different height levels in order to identify in wich level with major diversity and amount of antagonistic isolates. Four, out of five, of the endophytes with inhibitory activity against *F. oxysporum* were recovered from the low level.

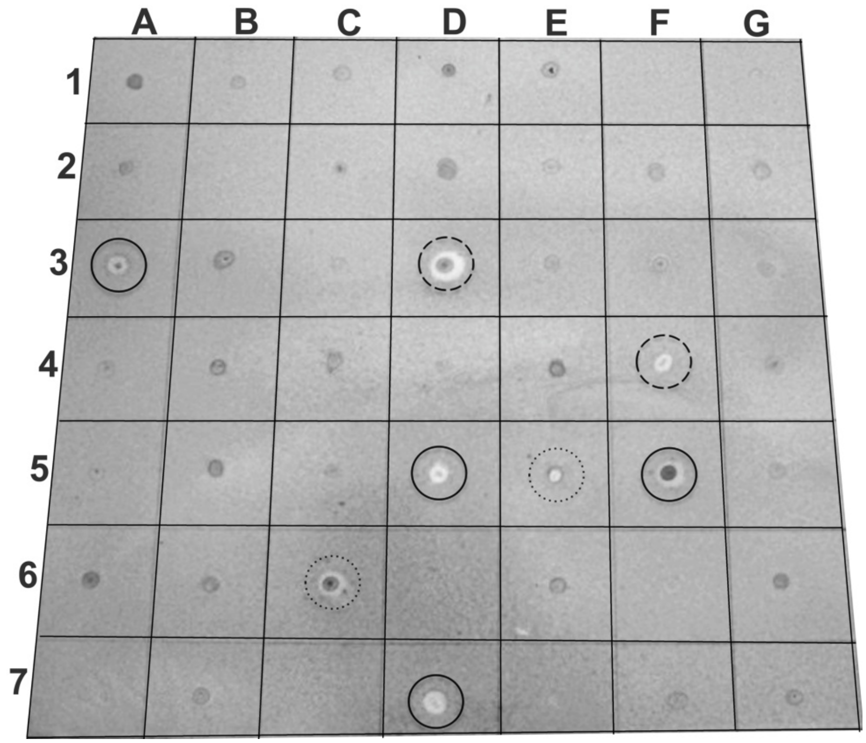

Figure 4. Bioautography of endophytes-derived EtOAc extracts against *F. oxysporum* conidia. Continuum lines: Procloraz 40ng (control). Medium dashed line: Supernatant medium of EtOAc extract from *C. globosum* F211_UMNG. Highly dashed line: Supernatant medium of EtOAc extract from *M. guilliermondii* F281_UMNG.

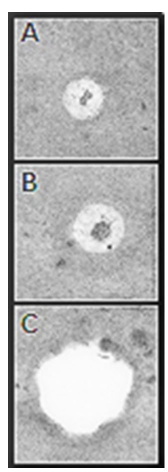

Figure 6. Bioautography of F211_UMNG extract and most active fraction against *F. oxysporum* conidia

A: Fraction #14, B: Extract 211 INI, C: Sportak 40 ng.

From the recovered isolates, fungi like *Alternaria* sp., *Aspergillus* sp., *Chaetomium* sp., *Epicoccum* sp., *Fusarium* sp., *Pestalotiopsis* sp., *Phomopsis* sp., *Xylaria* sp., among others were identified by their morphological traits and have been previously reported as common endophytes in other plants (Kjer et al., 2009; Liu et al., 2015; Zhang et al., 2013; de Lima et al., 2012; Demers et al., 2015; Yang et al., 2015; Murali et al., 2006; Davis et al., 2003). El-nagerabi et al. 2013 also found a high diversity of fungal species in leaves and stems of *Boswellia sacra* (Burseraceae), were the most dominant genera were *Alternaria* and *Aspergillus,* all also isolated in this work. However *Chaetomium* also was found in a relative high proportion (26.3%) represented by two species *C. globosum* and *C. spirale*. Screening works in *Boswellia serrata* the dominant endophytes were *Myrothecium verrucaria* and *Phoma* sp. (Sunayana, & Prakash, 2012) which were also isolated in our samples.

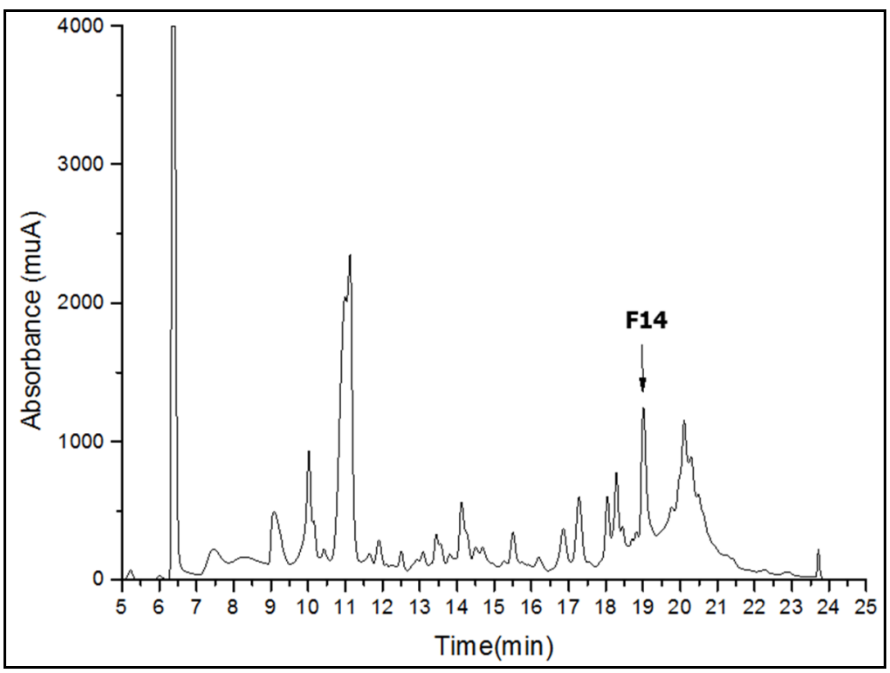

Figure 5. Chromatografic profile of EtOAc extract from F211_UMNG in YES medium. F14+arrow indicates fraction #14.

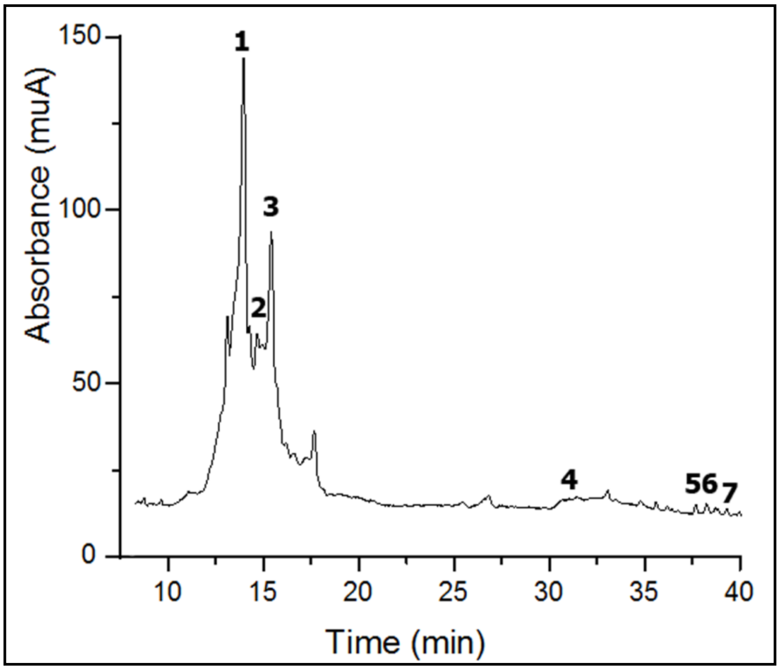

Figure 7. RP-HPLC-DAD chromatogram of fraction #14. Numbers indicate the tentatively identified compounds by MS data.

The 355 endophytes isolated in this work were evaluated by dual culture method against *F. oxysporum* and only five endophytes presented inhibition against *F. oxysporum* presumably by metabolite production because they inhibited the extension on the colony without mycelial contact and reduced the area of the phytopathogen at least 40% (Table 1, Fig. 2). Antagonistic endophytes were identified by amplification of a 570 bp (ITS) region as *C. globosum*, *Meyerozyma* spp., *Phyllosticta* spp., *T. amestolkiae,* and *X. grammica,* endophytes that have been previously reported with antibiotic activity (Zhang et al., 2013; Joel & Bhimba et al., 2013; Sakai et al., 1970; Baayen et al., 2002; Bara et al., 2013; Song et al., 2014, Okane et al., 2102). With the aim of discern the active component that possibly produce the inhibition of *F. oxysporum*, EtOAc-soluble extracts from antagonistic endophytes were prepared and tested against *F. oxysporum.* The extract from the isolate *C. globosum* F211_UMNG at a concentration of 30 mg generated inhibition of the growth of *F. oxysporum* (Fig. 4). This extract was further fractionated by preparative RP-HPLC to identified the bioactive compound. Once the 20 resulting fractions were collected and concentrated, they were also evaluated by direct bioautography. The fraction # 14 thus inhibited the growth of *F.*

oxysporum at 30 mg in the direct bioautography (Figure 5) at comparable level to that of crude extract. LC-MS analysis of fraction #14 revealed the presence of three main compounds. Based on available literature on the *Chaetomium* genus, mostly in *C. globosum,* seven signals defined by mass spectrum analysis were found to have the same m/z value to that reported (Table. 2). Nevertheless, five isomers previously reported on the *Chaetomium* genus matched with the m/z value detected at 38 min (peak 5 Fig. 7) and they cannot be therefore differentiated according to the known MS limitations (Figure 7). Previous studies found that *C. globosum* synthetized several molecules like chaetoglobosins, epipolythiodioxopiperazines, azaphilones, xanthones, anthraquinones, chromones, depsidones, terpenoids, and steroids, among others. These types of compounds have showed antitumor, cytotoxic, antimalarial, enzyme inhibitory, antibiotic, and other activities (Zhang et al., 2012). In the present study the cladosporin, chaetoatrosin A and chaetoviridin A (Fig. 8) were tentatively identified to be active against *F. oxysporum*, in the EtOAc extract of *C. globosum* F211_UMNG. These compounds were previously reported with antifungal activity (Wang et al., 2013; Park et al,. 2005; Hwang et al., 2000). The compound chaetoatrosin A acts as inhibitor of the chitin synthase II, while the chaetoviridin A inhibits the cholesteryl ester transfer protein (CETP) (Tomoda et al., 1999). The action of cladosporin is no fully understood but it has been reported that exhibited lysyl-tRNA synthetase inhibition in *P. falciparum* (Hoepfner et al., 2012) and its mode of action is different to that than affect the ß-tubuline assembly in mitosis (Wang et al., 2013). The combination of the modes of action of the identified molecules might rationalize the observed growth inhibition of *F. oxysporum* in the *in vitro* and bioautography test.

Table 2. Tentatively identified compounds isolated from other *Chaetomium* strains as constituents of fraction #14 . (No) column identifies the peak in the Figure 7.

No	Retention time (min)	Molecule	Isolated from	Molecular weight	Referenc e
1	13.0	Cladosporin	*Chaetomium globosum* CCTCC AF 206003	292,13	Wang et al., 2006

2	14.6	Unknown	-	-	-
3	15.5	Chaetopyranin	*Chaetomium globosum*	339.16	Wang et al., 2006
4	31.0	chaetoatrosin A	*Chaetomium atrobrunneum* F449	262,08	Hwang et al., 2000
5	38.0	chaetomugilin C	*Chaetomium globosum*	432,13	Muroga et al., 2009
		chaetomugilin N	*Chaetomium globosum*	433,9	Muroga et al., 2009
		epi-chaetoviridin A	*Chaetomium globosum*	433,9	Borges et al., 2011
		chaetoviridin A	*Chaetomium globosum*	433,9	Borges et al., 2011
		4'-epi-chaetoviridin A	*Chaetomium globosum*	433,9	Borges et al., 2011
6	38.4	Differanisole/Differanisole A	*Chaetomium* strain RB-001	278,01	Oka et al., 1985
7	39.7	Unidentified, Reaxys Registry Number: 19364999	*Chaetomium globosum* ZY-22	460,7	Qin et al., 2009

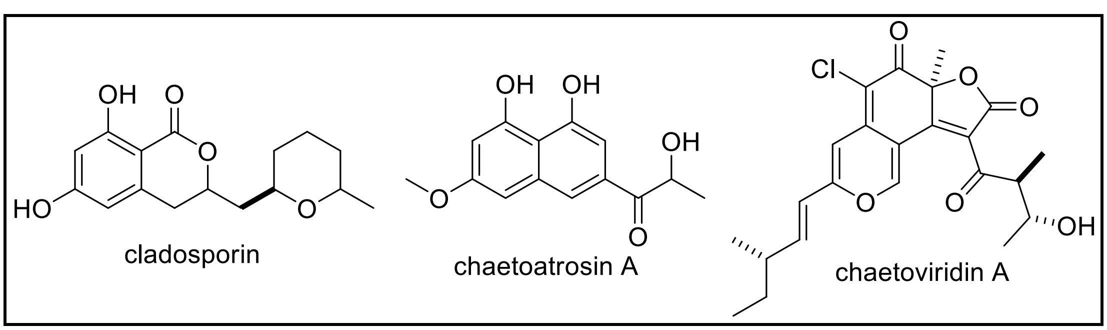

Figure 8. Structures of the identified compounds in the most active fraction from EtOAc-soluble extract of *C. globosum* F 211_UMNG.

In conclusion, the present study were isolated and identified five endophytes that act as antagonists of *F. oxysporum* under *in vitro* conditions. The isolate *C. globosum* F211_UMNG-derived extract inhibit the growth of *F. oxysporum,* possibly by at least three molecules with different mode of action, implying its possible application in control schemes of *F. oxysporum* (Michielse & Rep, 2009). Confirmation of the results in *in vivo* test is required; where the endophyte *C. globosum* and the purified molecules could be evaluated in the control of the disease caused by *F. oxysporum*.

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<u>CAPITULO 2</u>

Screening of Endophytic *Bacillus* sp. Isolated from *Bursera simaruba* (Burseraceae) with Antifungal Activity against *Botrytis cinerea* and *Fusarium oxysporum.*

Abstract

Phytopathogenic fungi *Botrytis cinerea* and *Fusarium oxysporum* are mayor threats in several crops. These pathogens are predominantly controlled by chemically synthesized fungicides whose constant use could result in environment detriment, and increased genetic resistance to these molecules, preventing an effective control of these organisms in field. Then, alternative methods for controlling pathogenic fungi rare being sought, resulting endophytes as a viable alternative to chemically synthesized fungicides due their interactions with others microorganisms and the reported roles in plant defense. In this work bacterial endophytes were isolated form *Bursera simaruba*, a tree that has been related to endophytes of remarkable potential in agriculture as *Muscodor yucatensis*. Five endophytes were isolated from leaves: four were identified as *Bacillus* sp. and one as *Pseudomonas geniculata.* Effects of these endophytes on *B. cinerea* and *F. oxysporum* were tested *in vitro* by using dual culture technique. Isolate B3_UMNG, identified as *Bacillus* sp., was the most effective isolate for inhibiting the colonial growth of both pathogens (61% for *F. oxysporum* and 79% for *B. cinerea*). The ethyl acetate extract from a B3_UMNG culture was therefore characterize by LC-MS. Multivariate analysis of the results along with literature comparison led to the tentative identification of lichenysin, a surfactin produced by *Bacillus*, as the main compound responsible of the antifungal activity.

Keywords: *Bacillus, secondary metabolism, antifungal, lichenysin, Multivariate analysis*

Introduction

Fungi *Botrytis cinerea* and *Fusarium oxysporum* are important phytopathogens that have a broad host range, and cause important economic loses in several crops (Bosland, 1988; Vrind, 2003). Common management of these pathogens relies on the use of synthetic pesticides, which results in an expensive and, for several pathosystems, cost ineffective control method due to development of genetic resistance by the pathogen(Myresiotis et al., 2007) and environmental detriment (McMahon et al., 2012). Lack of new molecules to the control of plant and human bacterials pathogens is in fact an important concern that could result in a serious treat to health and food security of the next generations (Gross, 2014). An alternative solution to these problems comes from ecology of microorganisms, since some individuals posses strategies to compete out competitors off their niches, such as producing growth suppressants, antibiotics, antimicrobial proteins or anti-nutritional compounds, which reduce competitor's fitness (Glare et al., 2012). These observations have driven investigations into employ such organisms as biological control agents of plant pathogens, or as sources of antimicrobial molecules (Hynes & Boyetchko, 2006).

Microorganisms, like fungal and bacterial endophytes, are an abundant and almost unexplored source of metabolites with possible use in agriculture (Kanchiswamy et al., 2015). Although their richness is still underestimated, the tropics have been considered as hyperdiverse regions and regarding the estimation of at least one endophyte inhabit every land plant (Arnold et al., 2000), the potential chemical diversity of the tropical endophytes could be an abundant source of active molecules with antimicrobial activity against phytopathogens (Strobel & Long, 1998). Among bacterial taxa found as endophytes, it is frequent to find species belonging to the *Bacillus* genus, which is known for producing several metabolites with agronomical and industrial uses (Stein 2005). In the recent years, various *Bacillus* sp. strains like *B. subtilis, B. amyloliquefaciens, B. cereus, B. pumilus,* and *B. licheniformis* were used as potential biocontrol agents against different *Fusarium* sp. and *Botrytis* sp. (Corrales et al., 2012; Zhao et al., 2014; Ajilogba et al., 2013, Kim et al., 2007; Sadfi-Zouaoui et al., 2008; Lee et al., 2006; Audenaert et al., 2002). For example, *B. subtilis SG6* produces a mix of chitinases, fengycins and surfactins that inhibit the growth of *F. graminearum* up to 100 % under *in vitro* conditions and reduced disease incidence (DI) up to 77% (Zhao et al., 2014). Growth inhibition of *B. cinerea* has also been reported in strawberry were formulation of *B. licheninsis* has reduced disease symptoms up to 81% in field conditions (Kim et al., 2007).

The botanical family Burseraceae is composed by resinous trees and is predominantly found in the tropics. Among its members are the myrrh (*Commiphora myrrha*) and the frankincense (*Boswellia* sp.). In this family, the *Bursera* genus has been studied for the presence of active compounds, such as terpenoids and phenolics, with anti-inflammatory, antimicrobial, antioxidant, antitumoral and insect repellent properties (McDoniel & Cole, 1972; Robles et al., 2005). In this genus species *Bursera simaruba* has been reported the presence of endophytes with antimicrobial activity, e.g. *Muscodor yucatanensis* (Macías-Rubalcava et al., 2010).

Given this background the aim of the present study was the exploration of the bacterial endophytes isolated from *B. simaruba* in Casanare, Colombia and to determine its ability to inhibit *F. oxysporum* and *B. cinerea* under *in vitro* conditions.

Methods

Bacterial endophytes sample and isolation

Individuals of *B. simaruba* were sampled in a tropical rain forest in Casanare, Colombia, during the 2013 winter season (april). Leaves from the bottom and middle part of two trees were collected and packaged in sealed bags and stored at 4ºC until use. Leaves were rinsed with tap water (x4) to remove any residues adhered to them. The washed material was superficially disinfected in turn with ethanol 70% during 1 min, NaClO 1% for 3 min and sterile distilled water (x4). Leaf printing on Nutrient Agar (NA) (Oxoid, UK) was done as control of disinfection of epiphytic organisms. Leaves were sectioned in explants of *ca.* 2 mm^2 and placed in NA for 6 days. The that bacteria grew out from the explants were recovered and stored in Nutrient Broth (NB) (Oxoid, UK) + glycerol 15% at -20 °C until reactivation.

DNA extraction and 16S rRNA gene amplification

A single colony derived from a 24h-old culture in NA was isolated and streaked on to 250 mL of NB follow by incubation in agitation, on an orbital shaker at 100 rpm, at 20°C for 24 hours. Total DNA for PCR amplification was obtained by boiling cells suspended in sterile distilled water for 10 min at 95°C. The 16S rRNA gene was amplified by PCR (denaturation step for 5 min at 95 °C; 35 cycles consisting of 60 s at 94°C, 60 s at 54°C and 120 s at 72°C; followed by 10 min at 72°C) using Taq Polymerase (Bioline, UK) and universal primers 27-F (5'-GAGTTTGATCCTGGCTCAG-3´) (Lane, 1991) and 1492-R (5'-CGGTTACCTTGTTACGACTT-3') (Turner et al., 1999). The amplicons were sequenced in Macrogen, Korea.

Phylogenetic analysis

The obtained nucleotide sequences were trimmed and verified manually to be assembled in BioEdit version 5.0.6 (Hall, 2001) and then isolates were identified using the EzTaxon server (http://www.ezbiocloud.net/eztaxon); Kim et al., 2012) on the basis of 16S rRNA sequence data. The closest sequences to the query were aligned with Clustal W (Thompson et al. 1997) and phylogenetic tree was inferred using MEGA version 6.6 (Tamura et al. 2007) by neighbor-joining method (Saitou and Nei 1987) with a bootstrap analysis of resampling data (x1000). The nucleotide sequences of the endophytes were submitted to GenBank database and the NCBI GenBank accession number was obtained.

Antifungal activity test

Stored bacteria were reactivated in NB by stirring during 24 h at 100 rpm and then cultured on NA at 25 °C. Pathogenic isolates of *B. cinerea* and *F. oxysporum* were obtained from our lab collection, and they were reactivated from monosporic cultures by streaking them on potato dextrose agar (PDA) (Oxoid, UK). A mycelial plug (3mm) taken from actively growing fungus colony form a 6 days old PDA culture was placed in the center of a PDA-containing Petri dish. Then, a bacterial endophyte was inoculated in two parallel lines, which were placed at each side of the center of the plug at a distance of 2.5 cm. As a control, a plug of fungus was inoculated without bacteria. Cultures were incubated during 6 days at 25°C prior evaluation. Area of the fungus colony, and the distance to the bacterial lines were recorded and compared against the control. This procedure was performed for each combination of fungus and bacteria and replicated three times. Data collected were analyzed by Tukey test ($p>0.05$) using the InfoStat software package (Di Rienzo et al., 2014).

Liquid chromatography coupled to mass spectrometry (LC-MS) analyses

Ethyl acetate-soluble (EtOAc) extracts were obtained from a 5–days-old culture of the endophytes in NB. Growth medium was centrifuged at 5000 rpm and then supernatant and pellet were collected. The supernatant was subjected to a liquid-liquid extraction with ethyl acetate at 3:1 ratio. The resulting extract was suspended in absolute ethanol and filtered through 0.2 um pore syringe filter prior injection in Shimadzu Prominence LC-MS 2020 equipment. The separation was achieved on a Synergi™ 4 µm Hydro-RP 80 Å, C-18 (150mm x 4.6mm) column. Formic acid 0.1% and Acetonitrile (HPLC grade, Merck) were used in a solvent mix ramp at a flow rate of 1 mL/min. The LC-derived data of all extracts was used as dataset to generate a Partial Less Square (PLS) regression in SIMCA software, v. 13.03.3 (Umetrics, Umeå, Sweden) with the aim of associate the composition with the antifungal activity. The obtained mass spectra data for each main detected compound in LC profiles were compared with literature for tentative identification. The mass spectrometry detector (MSD) consists of an electrospray ionization (ESI) and a detector with a triple quadrupole mass analyzer. The mass spectrometry method consisted of a scan in simultaneous positive and negative ionization with an acquisition time of 2-33 min, a mass range of 50-2000 m/z, a scan speed of 1667 u/s, an event time of 0.5 s, nebulizer gas flow of 1.5 L/min, 350 °C interface temperature and DL and 450 °C block temperature. The drying gas flow rate was 9 L/s. The analysis was monitored at wavelengths between 270 and 330 nm. Tentative identification of the major and minor metabolites in the extract was performed through the analysis of mass spectra based on the LC-MS data, complemented with the analysis of retention times and reported bacterial metabolites.

Results

After culturing 75 explants from different leaves, only five culturable bacterial endophytes were isolated. The total DNA was extracted and used to amplify the 16S rRNA gene with the 27f and 1492r primers for identification purposes. The resulting nucleotide sequences were deposited in the GenBank database and submitted to the EzTaxon server (http://www.ezbiocloud.net/eztaxon; Kim et al., 2012) (Table 1). Four isolates were identified to belong toe *Bacillus* genus, and one isolate (B2_UMNG) was identified as *Pseudomonas geniculata* ATCC 19374. The isolate B1_UMNG was related to the *Bacillus subtilis* group while the isolate B3_UMNG had a close match to *B. siamensis* belonging to the *B. amyloliquefaciens* group. The phylogenetic tree (Fig. 1) grouped the endophytes B3_UMNG, B4_UMNG and B5_UMNG near *B. amyloliquefaciens* group while B1_UMNG is near the *B. subtilis* group. The B2_UMNG endophyte was clustered with *P. geniculata*.

Table 1. Taxonomical identification of bacterial isolates using EzTaxon server and NCBI accession number.

Isolates	NCBI accession	The closet EzTaxon match	Identity (%)
B1_UMNG	KU058690	*Bacillus tequilensis* KCTC 13622	100
B2_UMNG	KU058691	*Pseudomonas geniculata* ATCC 19374	100
B3_UMNG	KU058692	*Bacillus siamensis* KCTC 13613	99.93
B4_UMNG	KU058693	*Bacillus methylotrophicus* CBMB205	100
B5_UMNG	KU058694	*Bacillus methylotrophicus* CBMB205	100

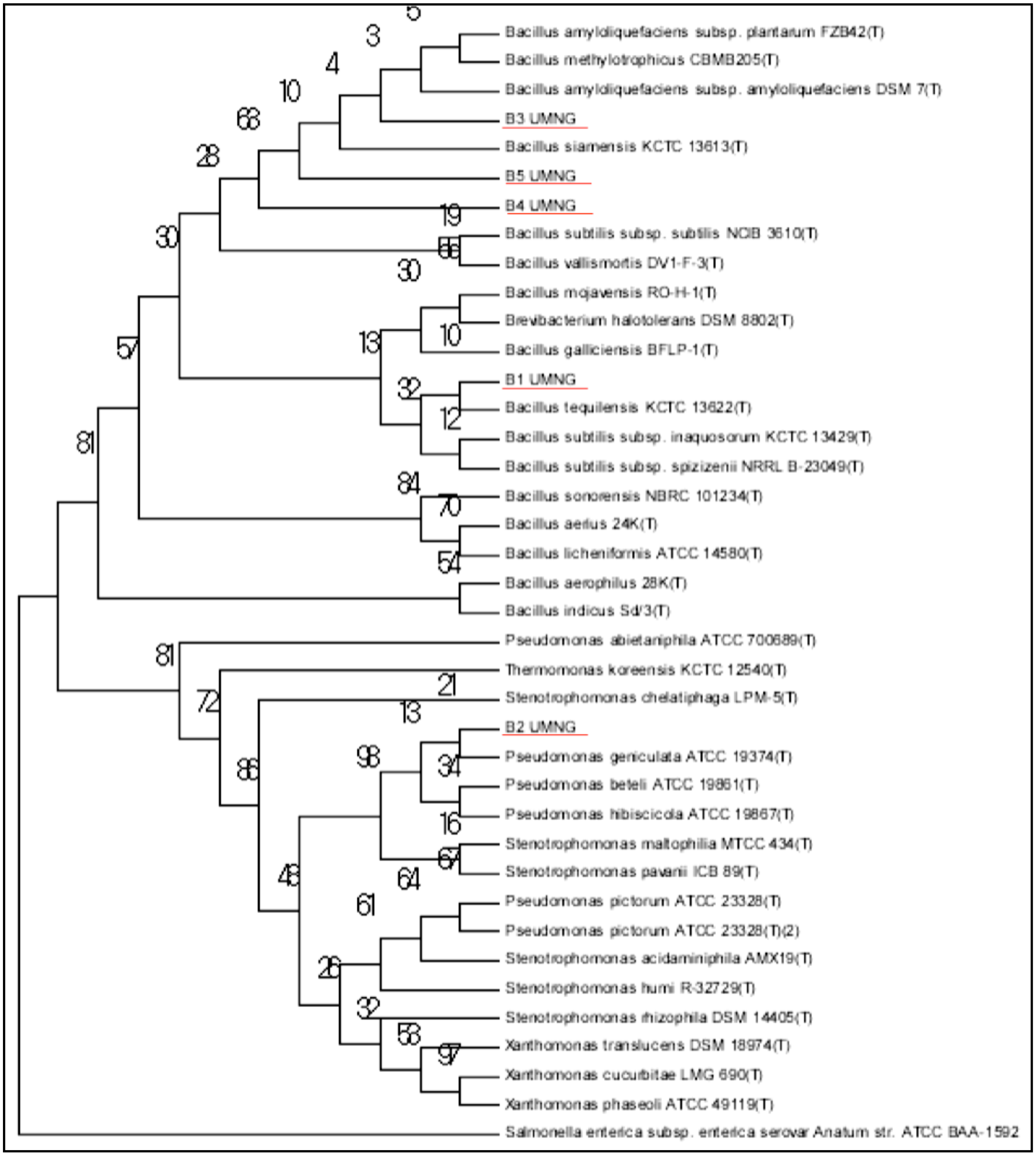

Figure 1. Dendrogram showing the phylogenetic relationship of bacterial endophytes based on 16S rDNA sequences. Phylogenies were inferred using neighborjoining analysis and trees generated in MEGA 6.06 software. Numbers at branch points indicate bootstrap values. Sequence of *Salmonella enterica subsp. enterica* serovar Anatum Str.

ATCC BAA-1592 was used as outgroup reference in the tree. Isolates underlined identified endophytes isolated in this work.

Antifungal test

All bacterial isolates inhibited *F. oxysporum* and *B. cinerea* colonial growth (Fig. 2 and 3), however, *B. cinerea* was the most susceptible pathogen to the presence of the bacterial endophytes. The isolate that exerted the most marked growth restriction in *inbinh* was B3_UMNG with an inhibition of 79 +/-1.1%, while the other endophytes oscillated between 71+/-4.5 % and 57+/-4.6%.

The inhibition of *F. oxysporum* was similar (Fig. 2), however was less affected by the present of the endophytes than *B. cinerea*, with inhibition values between 61+/-0.9 % and 33%+/-1.2 (Fig. 2).

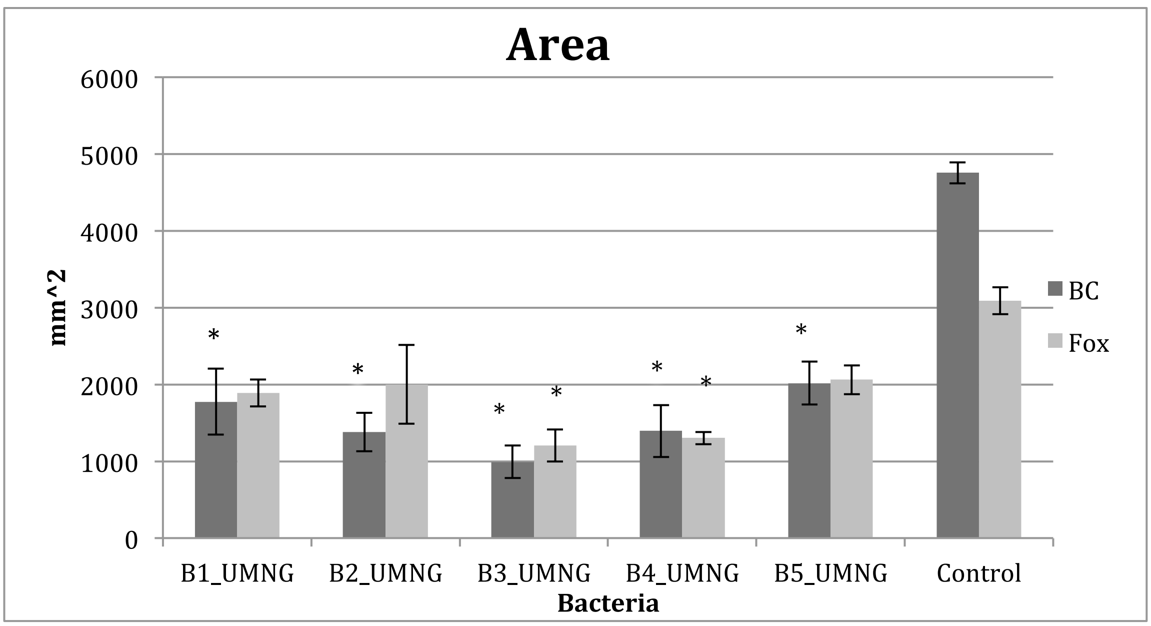

Figure 2. In vitro effect of the bacterial endophytes, on the colonial area of growth of *F. oxysporum* (Fox) and *B. cinerea* (BC).

* Statically different from control according to Tukey test (p> 0.05).

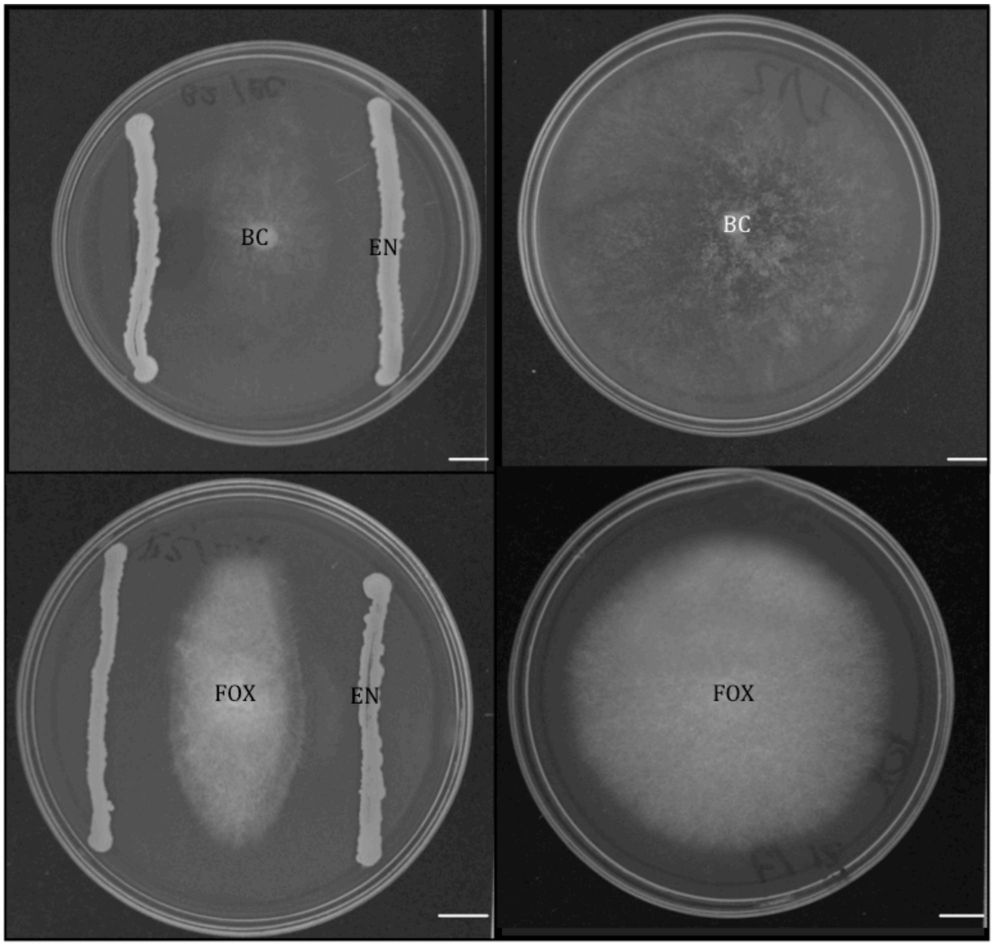

Figure 3. *B. cinerea* (BC) and *F. oxysporum* (FOX) challenged against bacterial endophyte B3_UMNG (EN) (Left) compare with the control (right).

Chemical analysis

Two EtOAc extracts were prepared from all five endophytes cultured in Nutrient broth (NB): from the intracellular content represented by the pellet (P) (obtained after centrifugation of the culture) and extracellular part represented by the supernatant (S) (Fig. 4). The control extract of the intracellular (P) and extracellular (S) parts presented relatively strong signals at 5 and 15 min, signals that are also exhibited in the cultured endophyte-derived extracts that are represented by the peaks 11, 12 and 14 (Fig. 4). Strong signals were generated by majority of the extracts (compounds with polar characteristics) (Fig. 4, peaks 1-4), and in the middle zone between 10 and 15 min (Fig. 4, peaks 5-10).

An OPLS-DA regression was performed to discriminate chromatographic signals related to the extract obtained from the most active endophytes. The LC-derived profile data at 270 nm was introduced into an OPLS-DA model. The resulting score plot (t1 vs. t1) clustered the extracellular extracts of the most active bacteria (B3_UMNG and B4_UMNG) in the same group (Fig. 5), suggesting an important correlation between the antifungal effect and the extracts composition.

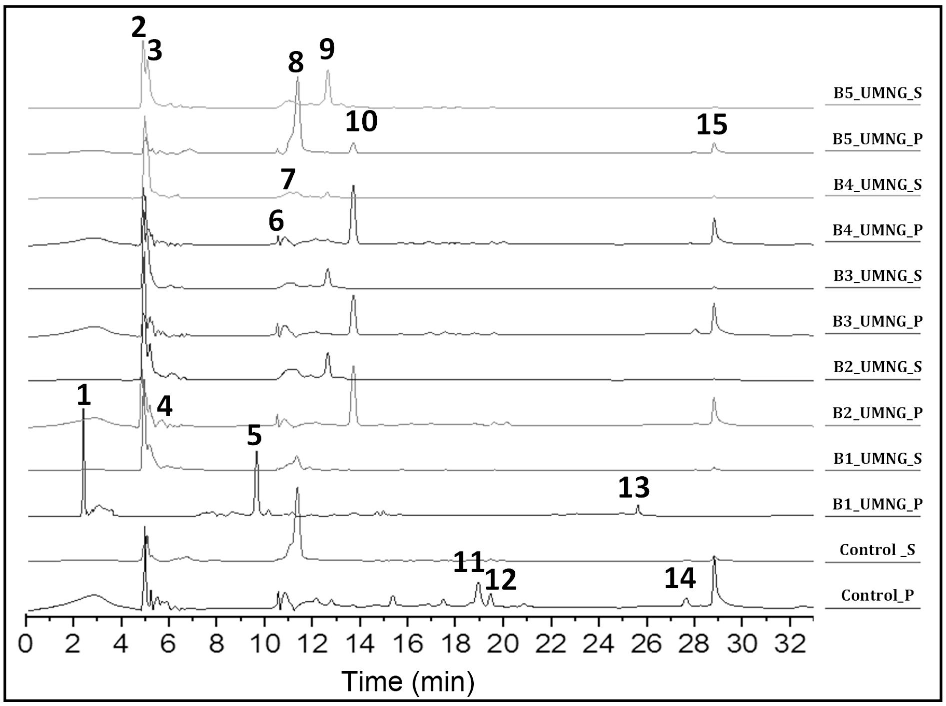

Figure 4. LC-derived chromatogram (270 nm) of the intracellular (P) and extracellular (S) extracts of endophytes of *B. simaruba.*

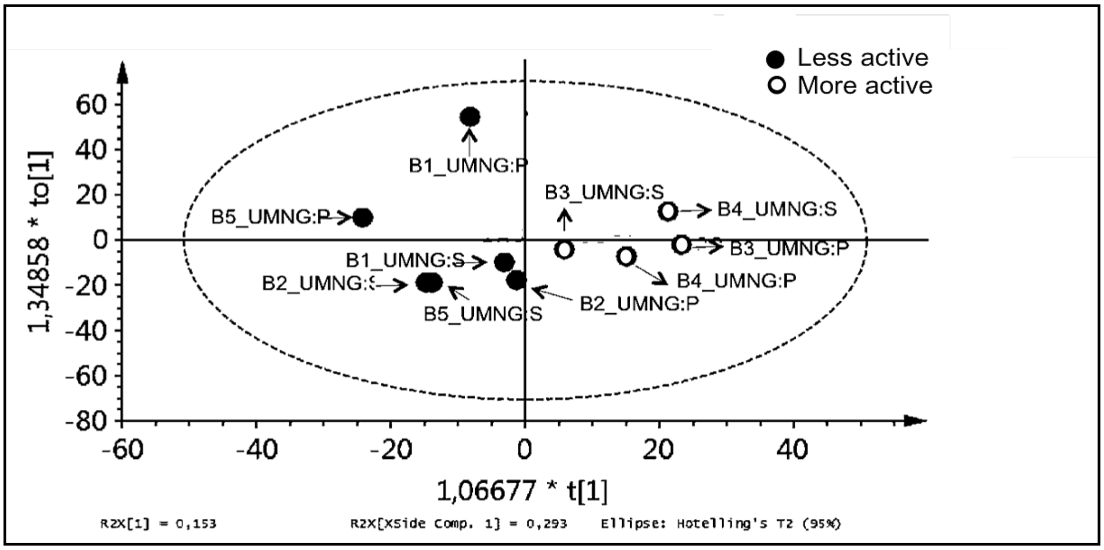

Figure 5. OPLS-DA model-derived score plot on chromatographic data of bacterial extracts discriminated by antifungal activity.

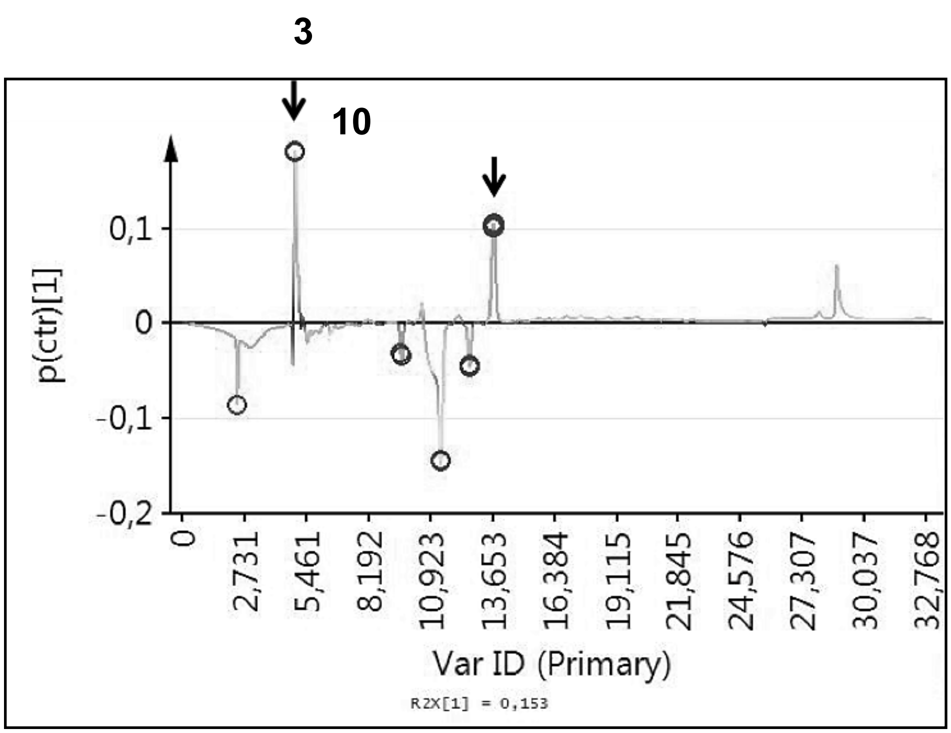

Figure 6. S-line derived from the OPLS-DA analysis. The X-axis (Var ID) corresponds to the retention time in the chromatogram. Arrows indicate those hits correlate with inhibitory activity that correspond to peaks 3 and 10.

The supernatant EtOAc-soluble extract of the B3_UMNG endophyte was selected for LC-MS analysis due to its outstanding inhibition of *B. cinerea* and *F. oxysporum* in relation to the other endophytes (Fig. 2). The MS results were analyzed comparing values with data previously reported in literature for the *Bacillus* genus. At least six compounds previously isolated in *Bacillus* genus were tentatively identified in the extract by MS data (Table 2). At 15 min peak 10 was highlighted by the OPLS-DA model as the region which possibly contain the compound or mix of compounds that exerted the antifungal activity (Fig. 6). In this region a characteristic cluster of m/z values for lichenysin, a non-ribosomal peptide form the surfactin family (Grangemard et al., 1999), were detected (Table 2). Other m/z representatives of other non-ribosomal peptides belonging to the iturin family were also obtained (Table 2- 8.2, 9.3 and 19.2 min).

Table 2. Comparison of *m/z* from the EtOAc extract of B3_UMNG and molecules identified and isolated from other *Bacillus* strains that shares similar m/z values.

Retention time (min)	Identified Compound	Peak #	$[M+H]^+$ (m/z)	Reference
8.2	iturin A, iturin A2	[a]	1044.1	Kong et al., 2010
9.3	anteiso-C17 bacillomycin D	5	1074.3	Tanaka et al., 2014
10.4	ethyl homononactyl nonactate	6	429.75	Han et al., 2014
10.4	gageomacrolactin 3	6	568.05	Tareq et al., 2013
15.3	lichenysin G15	10	107.35, 1021.3, 135.35	Grangemard et al., 1999
19.2	mixirin B	[a]	1000.25	Zhang, et al., 2004

[a] Signal not detected by the DAD but detected with the MSD.

Discussion

Endophytes are microorganisms living inside plants without inducing disease (Arnold et al., 2000). They interact with their hosts in different ways and the interaction confers benefits or advantages to the host, in some scenarios. In this work *B. simaruba* leaves from Casanare, Colombia, were sampled and bacterial endophytes recovered, in order to explore their potential use in the control of *B. cinerea* and *F. oxysporum,* two important pathogens of several crops (Bosland, 1988; Vrind, 2003). Only five bacterial endophytes were recovered from 75 explants obtained from 35 leaves evaluated. This low recovery rate could be explained because *B. simaruba* leaves are highly resinous, and produce several secondary metabolites like triterpenes, steroids, bilignans, podophyllotoxin-like lignans and flavonoids (Cicció& Rosales, 1995; Maldini et al., 2009; Peraza-Sánchez & Peña-Rodríguez, 1992; Peraza-Sánchez et al., 1995) which could have prevented the successful colonization of other bacterial endophytes.

The sequence analysis of 16S rRNA gene of endophytes showed that four strains belonged to the *Bacillus* genus, and one was identify as *P. geniculata.* The isolate B1_UMNG was related to the *Bacillus subtilis* group that compiles six different subspecies and species (*B. subtilis* subsp. *subtilis, B. subtilis* subsp. *spizizenii, B. subtilis* subsp. *inaquosorum, B. mojavensis, Brevibacterium halotolerans* and *B. tequilensis* (Logan & Berkeley, 1984). Similarly, the isolate B3_UMNG had a close match to *B. siamensis* belonging to the *B. amyloliquefaciens* group that also comprises three organisms (*B. amyloliquefaciens* subsp. *amyloliquefaciens, B. amyloliquefaciens* subsp. *plantarum,* and *B. siamensis).* Although the molecular approach to identified bacteria according to the variability of the 16S rRNA gene has proved to be useful to separate species, in the *Bacillus* genus many of the species share a remarkably high level of similarity of the16S rRNA gene sequence (often 99% or greater), phenotypic and biochemical characteristics (Hutsebaut et al., 2006). Therefore, in order to asses a reliable molecular identification in the *Bacillus* genera is required a multilocus phylogenetic analysis (Rooney et al., 2009). *Bacillus* genus predominated in this sample from *B. simaruba,* this trend has been observed in others plants such as *Beta vulgaris*

(Jacobs et al., 1985), *Gossypium hirsutum* L. (Misaghi & Donndelinger, 1990), *Solanum tuberosum* L. (Hollis, 1951) and *Zea mays* L. (Lalande et al., 1989).

The results in the present work indicated that two isolates from Bacillus genera (B3_UMNG and B4_UMNG) exhibited the highest antifungal effect on the mycelium growth of *B. cinerea* and *F. oxysporum*, probably by the action of diffusible metabolites (Fig. 3), a common behavior of *Bacillus* (Stein 2005). Thus, a possible explanation for such a behavior may be due to one or more antifungal compounds produced by endophyte. Several *Bacillus* species have been reported as producers of antifungal lipopeptides as surfactins, iturins and fengycins (Ongena & Jacques, 2008) and other metabolites like siderophores (Yu et al., 2011) or bacilysin (Phister et al., 2004) among others. In order to compare between the metabolite production by the isolates, EtOAc extracts were characterized by LC (Fig. 4) and some signalsas 2, 3 and 15 (Fig. 4) were identified as common for all extracts but other signals as 5-10 and 13 (Fig. 4) were found to be restricted to some extracts.

To correlate the chemical composition of the extracts and its antagonistic effects, LC data was used as data input to construct an OPLS-DA model discriminating by the activity. The resulting score plot (R^2= 0.446 at 95%) exhibited a clustering according the discriminant parameter, indicating therefore a good correlation (Fig. 5). The S-line plot (Fig. 6) derived from the OPLS-DA analysis highlighted two hits identified in the chromatogram in the 5.8 and 15.3 min, which could be considered as bioactive compounds.

The B3_UMNG-derived extract was analyzed by LC-MS because of its greatest activity against *B. cinerea* and *F. oxysporum*. The MS results showed at least four compounds possessing experimental *m/z* values to be similar to lipopeptides reported previously in literature for *Bacillus* sp. (Table 2). Iturin, bacillomycin D and mirixin B are lipopeptides from the Iturin family and have been previously described as potent antifungal, antimicrobial and cytotoxic agents with great promissory potential in industry, medicine and agriculture (Ongena et al., 2008; Kong et al., 2010; Tanaka et al., 2014;

Zhang, et al., 2004). Peak 10 (Fig. 4) exhibited characteristic *m/z* values for lichenysin (Grangemard et al., 1999)(Table 2). This compound is a lipopeptide from the surfactin family produced by *B. lycheniformis* that seem to be synthesized under aerobic and anaerobic conditions (Javaheri et al., 1985); in addition, biosurfactant, antibiotic and antifungal properties have also been described (Käppeli & Fiechter, 1991). *Bacillus* lipopeptides are well known as potent antibactibacterial and antifungal molecules with strong antagonistic activities against various phytopathogens (Ongena & Jacques, 2008) like *Pseudomonas syringae, Pythium ultimum, Xanthomonas axonopodis pv.* glycines and *Sclerotinia sclerotiorum* have been successfully inhibited by lipopetides produced by *Bacillus* sp. strains (Alvarez et al., 2012; Bais et al., 2004; Onenga et al., 2005; Preecha et al., 2010). For example, surfactin produced by *B. licheniformis,* cause hyphal swellings and growth inhibition on *Magnaporthe grisea* (Tendulkar et al., 2007). The mode of action of these molecules seems to be determined by the amphiphilic character and their affinity for lipid bilayers (Deleu et al., 2008).

The bacterial endophytes are a potential source of metabolites for biological control agents. The use of metabolites is an alternative to synthetic agrochemicals for controlling fungal pathogens in agricultural production. (Glare et al., 2012). The *Bacillus* genus has been widely used in agriculture as biopesticides (Ongena et al., 2008) and its use as source of antifungal compounds is still to be assessed. We report endophytes from *B. simaruba* identified as *Bacillus* sp. with antifungal activity against *F. oxysporum* and *B. cinerea.* We thus demonstrated the potential of some endophytes of a tropical tree as source of antifungal metabolites that could be used in agriculture as biocontrol agents or source of biopesticides molecules.

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